Dual regulation of *zwf-1* by both 2-keto-3-deoxy-6-phosphogluconate and oxidative stress in *Pseudomonas putida*

Juhyun Kim,¹ Che Ok Jeon² and Woojun Park^{1,3}

Correspondence

Woojun Park
wpark@korea.ac.kr

¹Division of Environmental Science and Ecological Engineering, Korea University, Anam-Dong 5 Ga, Seoul, Republic of South Korea

²Department of Life Science, Chung-Ang University, Seoul, 156-756, Republic of South Korea

³Environmental Biotechnology National Core Research Center, Gyeongsang National University, Jinju, Republic of Korea

Northern blot analysis and a GFP-based reporter assay showed that *zwf-1*, which encodes glucose-6-phosphate dehydrogenase, was highly induced when *Pseudomonas putida* KT2440 was cultured in minimal medium containing glucose or gluconate. However, *zwf-1* expression was not detected in the presence of pyruvate or succinate. The use of a knockout mutant of HexR, a putative transcription regulator, resulted in constitutively high expression of *zwf-1*, regardless of the carbon source. An electrophoretic mobility shift assay showed that HexR protein binds to the *zwf-1* promoter region and that HexR binding is inhibited by 2-keto-3-deoxy-6-phosphogluconate (KDPG). Despite the presence of gluconate, the *edd* mutant (non-KDPG producer) was not able to induce the *zwf-1* gene. The *eda* mutant (KDPG overproducer) featured a constitutively high level of *zwf-1* expression. Interestingly, *zwf-1* was also highly expressed in the presence of oxidative stress-inducing reagents. The level of *zwf-1* induction in wild-type cells undergoing oxidative stress did not differ significantly from that observed with the *hexR* mutant under normal conditions. Interestingly, the *hexR* mutant was more tolerant of oxidative stress than the wild-type. Expression of *zwf-1* was induced by oxidative stress in the *edd* mutant. Thus, KDPG, a real inducer of *zwf-1* gene expression, was not necessary for oxidative-stress induction. *In vitro* binding of HexR to its cognate promoter region was diminished by menadione and cumene hydroperoxide. The data suggested that HexR might be a dual-sensing regulator of *zwf-1* induction that is able to respond to both KDPG and oxidative stress.

Received 8 May 2008
Revised 31 July 2008
Accepted 29 August 2008

INTRODUCTION

In *Pseudomonas* species, the Entner–Doudoroff (ED) pathway is used to metabolize glucose. Three pathways of initial glucose breakdown to 6-phosphogluconate (6PG) have been identified in *Pseudomonas* spp. (Fig. 1; del Castillo *et al.*, 2007b; Temple *et al.*, 1994). The first of these pathways is the glucose kinase pathway, in which glucose is transported into the cytoplasm by the glucose transport system. The second is the gluconokinase pathway, in which gluconokinase mediates the direct phosphorylation of gluconate or the oxidation of glucose to gluconate. Finally, there is the ketogluconate pathway, in which the formation of 2-ketogluconate is catalysed primarily by two consecutive periplasmic oxidation reactions involving

glucose dehydrogenase, encoded by the *gcd* gene, and gluconate dehydrogenase, encoded by the *gad* gene (Fig. 1). Through these pathways glucose is converted to 6PG, which is further metabolized into glyceraldehyde 3-phosphate and pyruvate by two ED enzymes, 6PG dehydratase (Edd) and 2-keto-3-deoxy-6-phosphogluconate (KDPG) aldolase (Eda) (Fig. 1; Cuskey *et al.*, 1985; del Castillo *et al.*, 2007b).

The genes for glucose metabolism in *Pseudomonas putida* are organized as several operons and are regulated by specific transcriptional regulatory proteins including HexR, GltR-2, PtxS and GnuR (del Castillo *et al.*, 2008). GltR-2 participates in the glucose transport system, PtxS regulates gluconate oxidation and GnuR controls the transport and phosphorylation of gluconate (del Castillo *et al.*, 2008).

HexR regulates two operons containing the *zwf* (glucose-6-phosphate dehydrogenase; G6PDH), *pgl* (6-phosphogluconolactonase) and *eda* genes; and the *edd*, *glk* (glucokinase)

Abbreviations: As, arsenic; CHP, cumene hydroperoxide; ED, Entner–Doudoroff; EMSA, electrophoretic mobility shift assay; G6PDH, glucose-6-phosphate dehydrogenase; KDPG, 2-keto-3-deoxy-6-phosphogluconate; MD, menadione; 6PG, 6-phosphogluconate; PQ, paraquat; ROS, reactive oxygen species.

Fig. 1. Carbohydrate metabolism in *P. putida* involves the oxidation of glucose to gluconate and 2-ketogluconate, and occurs in the periplasmic space. PEP, phosphoenolpyruvate; OM, outer membrane; PG, periplasmic space; IM, inner membrane.

and *gltR-2* (DNA binding response regulator GltR) genes (del Castillo *et al.*, 2008; Hager *et al.*, 2000; Petruschka *et al.*, 2002; Temple *et al.*, 1994). HexR also regulates the expression of glyceraldehyde-3-phosphate dehydrogenase, encoded by the *gap* gene (del Castillo *et al.*, 2008), and controls *zwf* in the ED pathway of *Pseudomonas aeruginosa* (Hager *et al.*, 2000). The central intermediate of glucose metabolism, 6PG, acts as an inducer in *P. aeruginosa* as well as in *P. putida* (Petruschka *et al.*, 2002; Temple *et al.*, 1990). However, this study showed that KDPG, and not 6PG, induces HexR in glucose metabolism.

In many bacterial strains, the *zwf* gene is induced in response to oxidative stress (Lundberg *et al.*, 1999; Park *et al.*, 2006; Pomposiello & Demple, 2001; Sung & Lee, 2007). NADPH, produced by G6PDH activity, is needed for reductive metabolic pathways and oxidative stress-damage repair reactions (Girò *et al.*, 2006; Lundberg *et al.*, 1999; Singh *et al.*, 2005). Deletion of the *zwf* gene increases bacterial sensitivity to oxidative stresses (Girò *et al.*, 2006; Lundberg *et al.*, 1999; Ma *et al.*, 1998). The *zwf* gene is activated by the SoxRS regulon of *Escherichia coli* (Demple, 1996; Greenberg *et al.*, 1990; Wu & Weiss, 1992). SoxR, a MerR-family transcription factor containing redox-active [2Fe-2S] centres, controls the SoxR regulon. SoxR is activated upon [2Fe-2S] oxidation and induces transcription of the divergently transcribed *soxS* gene (Hidalgo *et al.*,

1997). The *zwf* gene is one of the targets of SoxS in *E. coli* (Girò *et al.*, 2006; Pomposiello & Demple, 2001; Tsaneva & Weiss, 1990; Li & Demple, 1994) and is its direct transcriptional activator. The *P. putida* genome does not contain a clear SoxS homologue. We have previously shown that regulation of superoxide stress in *P. putida* differs from the SoxR paradigm previously described in *E. coli* (Park *et al.*, 2006). The *zwf-1* gene of *P. putida* KT2440 is strongly induced by oxidative stress reagents such as paraquat (PQ), menadione (MD) and nitric oxide, but its induction is not controlled by the SoxR system (Park *et al.*, 2006). Here, we show that HexR is a transcriptional repressor responding to KDPG in the context of glucose metabolism, and that it might also be a key regulator of the *zwf-1* gene under conditions of oxidative stress.

METHODS

Bacterial strains, culture conditions, and DNA manipulation.

Bacteria (Table 1) were grown at 37 °C (*E. coli*) or 30 °C (*P. putida*) in Luria-Bertani (LB) or mineral salts medium (M9) containing different carbon sources at the following concentrations: 10 mM succinate, 10 mM glucose, 10 mM gluconate, 10 mM fructose, 10 mM pyruvate or 0.5% (v/v) glycerol. Plasmid isolation, gel electrophoresis, transformation and PCR were performed using standard procedures (Ausubel *et al.*, 1999). Tetracycline (15 µg ml⁻¹) and kanamycin (50 µg ml⁻¹) were added to bacterial cultures

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype or relevant characteristics*	Source or reference
<i>P. putida</i> KT2440 strains		
<i>P. putida</i> KT2440	Wild-type	Laboratory stock
<i>P. putida</i> KT2440-R	Wild-type; Rif ^R	Laboratory stock
<i>P. putida</i> KT2440 (pRK _{zwf-1gfp})	Strain KT2440 harbouring pRK _{zwf-1gfp} ; Tc ^R , Rif ^R	This study
<i>P. putida</i> KT2440 (pRK221P _{zwf-1gfp})	Strain KT2440 harbouring pRK221P _{zwf-1gfp} ; Tc ^R , Rif ^R	This study
<i>P. putida</i> KT2440 (pRK136P _{zwf-1gfp})	Strain KT2440 harbouring pRK136P _{zwf-1gfp} ; Tc ^R , Rif ^R	This study
<i>P. putida</i> KT2440 (pRK67P _{zwf-1gfp})	Strain KT2440 harbouring pRK67P _{zwf-1gfp} ; Tc ^R , Rif ^R	This study
<i>P. putida</i> KT2440 (pRK19P _{zwf-1gfp})	Strain KT2440 harbouring pRK19P _{zwf-1gfp} ; Tc ^R , Rif ^R	This study
HexR–	Derivative of strain KT2440, insertion of pVIK-HexR; Rif ^R , Km ^R	This study
HexR– (pRK _{zwf-1gfp})	Strain HexR– harbouring pRK _{zwf-1gfp} ; Rif ^R , Km ^R , Tc ^R	This study
HexR– (pRK _{zwf-1gfp} -HexR CP)	Strain HexR– harbouring pRK _{zwf-1gfp} -HexR CP; Rif ^R , Km ^R , Tc ^R	This study
ΔEdd	edd: mini-Tn5-Km; Rif ^R , Km ^R	Duque <i>et al.</i> (2007)
ΔEdd(pRK _{zwf-1gfp})	Strain ΔEdd harbouring pRK _{zwf-1gfp} ; Rif ^R , Km ^R , Tc ^R	This study
ΔEda	eda: mini-Tn5-Km; Rif ^R , Km ^R	Duque <i>et al.</i> (2007)
ΔEda(pRK _{zwf-1gfp})	Strain ΔEda harbouring pRK _{zwf-1gfp} ; Rif ^R , Km ^R , Tc ^R	This study
<i>E. coli</i> strains		
BL21(HexR)	Strain harbouring pET28a-HexR	This study
Top10	<i>mcrA</i> , Δ(<i>mrr-hsdRMS-mcrBC</i>), <i>φ80lacZ</i> ΔM15	Promega
HB101	Strain harbouring pRK2013	Fredrickson <i>et al.</i> (1988)
S17-1 λpir	<i>trp</i> Sm ^R <i>recA thi pro hsdM</i> ⁺ RP4-2-Tc::Mu::Km Tn7; <i>hsdR</i> mutant	Simon <i>et al.</i> (1983)
Plasmids		
pRK415gfp	Broad-host-range vector, Tc ^R Mob ⁺	Yin <i>et al.</i> (2003)
pRK _{zwf-1gfp}	pRK415gfp carrying the KT2440 <i>zwf-1</i> full promoter region	This study
pRK221P _{zwf-1gfp}	pRK415gfp carrying the 221 bp upstream of the KT2440 <i>zwf-1</i> translation start point (promoter region)	This study
pRK136P _{zwf-1gfp}	pRK415gfp carrying the 136 bp upstream of the KT2440 <i>zwf-1</i> translation start point (promoter region)	This study
pRK67P _{zwf-1gfp}	pRK415gfp carrying the 67 bp upstream of the KT2440 <i>zwf-1</i> translation start point (promoter region)	This study
pRK19P _{zwf-1gfp}	pRK415gfp carrying the 19 bp upstream of the KT2440 <i>zwf-1</i> translation start point (promoter region)	This study
pRK _{zwf-1gfp} -HexR CP	pRK415gfp carrying the KT2440 <i>zwf-1</i> full promoter region and the <i>hexR</i> region	This study
pVIK112	R6KoriV, suicide vector, LacZ translation fusion vector, Km ^R	Kalogeraki & Winans (1997)
pVIK- <i>hexR</i>	Partial region of <i>hexR</i> gene cloned into pVIK112	This study
pRK2013	Helper plasmid for triparental mating	Fredrickson <i>et al.</i> (1988)
pET28a(+)	Protein overexpression with His ₆ -tag	Novagen
pET28a(+)-HexR	pET28a carrying the KT2440 <i>hexR</i> region	This study

*Rif^R, Km^R, Tc^R, resistance to rifampicin, kanamycin and tetracycline, respectively.

when necessary. The *edd* and *eda* mutants were purchased from Bio-Illiberis R&D. The KDPG substrate was provided by Matthew J. Walters (Duke University).

Northern blot analysis. Total RNA was isolated from 3 ml of exponentially growing cells using an RNeasy kit (Qiagen) following the manufacturer's instructions. RNA concentration was determined by measuring A₂₆₀ using 4–5 µg total RNA per sample. The fractionated RNA was transferred to a nylon membrane (Schleicher & Schuell) using a Turboblotter (Schleicher & Schuell), and the amounts of *zwf-1*, *zwf-2*, *zwf-3* and *edd* mRNA were determined by hybridizing the membrane with α-³²P-labelled probe specific for each gene (TaKaRa Bio). To generate the specific probes, the following primers were used: kt *zwf-1* Pp1, kt *zwf-1* Pp2, kt *zwf-2* Pp1, kt *zwf-2* Pp2, kt *zwf-3* Pp1, kt *zwf-3* Pp2, kt *edd* Pp1 and kt *edd* Pp2 (Table 2).

Cloning procedures and construction of bacterial strains. A broad-host-range promoter probe vector, pRK415gfp (Yin *et al.*, 2003), was used to construct a reporter plasmid, pRK_{zwf-1gfp}. Briefly, a 438 bp fragment from the *zwf-1* promoter was amplified using primers kt *zwf-1* pro2 and kt *zwf-1* pro3. To generate pRK_{zwf-1gfp}-HexR CP (a reporter plasmid containing the *hexR* plus the *zwf-1* promoter regions for complementation), the kt *zwf-1* pro1 and the kt *zwf-1* pro2 primers were used. The amplicon was cloned into the *EcoRI*/*Bam*HI cloning site of the pRK415gfp vector generating pRK_{zwf-1gfp}. The constructed plasmid was then introduced into *E. coli* Top10 by electroporation. Then, the pRK_{zwf-1gfp} plasmid was introduced by triparental conjugation into *P. putida* KT2440-R, thus creating *P. putida* KT2440 (pRK_{zwf-1gfp}). Conjugation was performed by triparental filter mating using *E. coli* Top10 (pRK_{zwf-1gfp}), *E. coli* HB101 (pRK2013) and *P. putida* KT2440-R as donor and recipient, respectively (Park *et al.*, 2003).

Table 2. Primers used in this study

Oligonucleotide	Sequence (5'–3')*
kt <i>zwf-1</i> pro1	CGCGAATTCGAGTCGACGCGTTTGTCT
kt <i>zwf-1</i> pro2	CGCGGATCCCCAGCAGCGGGTGTCC
kt <i>zwf-1</i> pro3	CGCGAATTCGCGGGTTGAGCAGGATGACTT
kt <i>hexR</i> KO1	CGCGAATTCGGTGCGCCCGGTGTAGGAA
kt <i>hexR</i> KO2	CGCGAATTCGGTAGAGGCCGACGATGAC
kt <i>zwf-1</i> Pp1	ATCCGCGACGAGAAGGTGAAAGTG
kt <i>zwf-1</i> Pp2	GGCCCTGCTCCTTGGTCATCAC
kt <i>zwf-2</i> Pp1	CCAGTGGCCGGGGTCATCTT
kt <i>zwf-2</i> Pp2	CCCATGGTCGGCAAAAT
kt <i>zwf-3</i> Pp1	GGCGAACC CGGTGGACTGAC
kt <i>zwf-3</i> Pp2	TCTCGCAAACGCTGATCTGGACAT
kt <i>edd</i> Pp1	GCCGCGAAGAGCTGTTGGAGTC
kt <i>edd</i> Pp2	GCCGGGATGTGCAGGGTGTGGT
kt HexR-OE1	CGCGGATCCGTGCGAAACCTCCTGGAACAG
kt HexR-OE2	CGCCTCGAGCGAGTCGACGCGTTTGTCT
kt <i>zwf-1</i> sp. pro1	TCGTACTGTCGGTGGGTTGA
kt <i>zwf-1</i> sp. pro2	GCAAGGTTGACACTGATT
kt <i>zwf-1</i> sp. pro3	CGCGAATTCGGTTTCGCACGCGGTCCATG
kt <i>Pzwf-1</i> pt1	CGCGAATTCCTCGTACTGTCGGTGGGTTGA
kt <i>Pzwf-1</i> pt2	CGCGAATTCATTATCCCTTGAAAACCGTGTG
kt <i>Pzwf-1</i> pt3	CGCGAATTCCTTAGGAAAGAACCAACATCA

*Restriction sites are underlined.

To construct the *hexR* mutant, a 313 bp fragment of its internal region was amplified by PCR using the kt *hexR* KO1 and kt *hexR* KO2 primers. The fragment was cloned into the *EcoRI* cloning site of the pVIK112 vector (Kalogeraki & Winans, 1997), generating pVIK-HexR. The constructed plasmid was then introduced by electroporation into *E. coli* S17-1 λ pir. Conjugation was performed by filter mating with *E. coli* S17-1 λ pir (pVIK-HexR) and *P. putida* KT2440-R (Lee *et al.*, 2006a) as the donor and recipient, respectively.

Quantification of GFP fluorescence. Bacterial cells at the exponential growth phase (OD₆₀₀ ~0.3) grown in M9 minimal medium supplemented with glucose, gluconate, glycerol, fructose, succinate or pyruvate, were collected using a microcentrifuge (15 800 g) and washed twice with PBS (137 mM NaCl, 10 mM phosphate, 2.7 mM KCl, pH 7.4). Then, both the OD₆₀₀ and GFP fluorescence intensity of the resuspended cultures were quantified using a microtitre plate reader (Victor3, Bio-Rad). This reporter strain expresses a stable GFP variant that absorbs light at 488 nm.

Overexpression of His–HexR. HexR was cloned from the genomic DNA of *P. putida* KT2440 by PCR (Kt HexR-OE1/OE2 primer pair used; Table 2) and inserted into pET28a(+). The recombinant plasmid was introduced into *E. coli* BL21 to obtain expression of the recombinant plasmid pET28a(+)-HexR. The transformed *E. coli* BL21, harbouring pET28a(+)-HexR, was cultured overnight in 5 ml LB medium supplemented with kanamycin at 37 °C with shaking at 220 r.p.m. The cells were then diluted 100-fold in 20 ml of the same medium and incubated for 3 h. IPTG (0.5 mM) was added, and the cells were incubated for an additional 4 h. The cells were harvested, washed twice with PBS, resuspended in Tris-Cl buffer (pH 7.5, containing 1 mM DTT), and lysed by sonication. Crude extracts were clarified by centrifugation at 10 000 g for 10 min at 4 °C, and the protein concentration was determined by the Bradford method using BSA as the standard.

Electrophoretic mobility shift assays (EMSAs). The *P_{zwf-1}* DNA probe was generated by PCR amplification using the *zwf-1* sp. pro1/*zwf-1* sp. pro2 primer pair (for the 136 region) and the *zwf-1* sp. pro2/*zwf-2* sp. pro3 primer pair (for the 221 region). The PCR product was dephosphorylated and labelled with [γ -³²P]ATP and T4 polynucleotide kinase. The reaction mixture (20 μ l final volume), containing the *P_{zwf-1}* probe, crude extract and loading buffer in 5 $\times$ binding buffer (50 mM Tris, pH 7.5, 10 mM MgCl₂, 50 %, v/v, glycerol, 10 mM DTT, and 375 mM KCl), was incubated for 20 min at room temperature. The resulting complexes were analysed by electrophoresis on 4 % polyacrylamide gels in 0.5 $\times$ Tris-borate/EDTA (TBE) buffer (5 $\times$ TBE = 1.1 M Tris, 900 mM borate, 25 mM EDTA, pH 8.3).

Oxidative stress sensitivity assay. Cells were grown in liquid LB medium overnight and diluted 100-fold in the same medium. After 3 h further incubation, serially diluted cells were spotted on LB agar with or without PQ (7 μ M), MD (1 mM) or arsenic (As) (10 p.p.m. arsenic oxide, Sigma).

Measurement of G6PDH activity. Cells grown under each set of conditions were lysed by sonication in 50 mM Tris buffer (pH 7.5). G6PDH activity was monitored using a spectrophotometer (340 nm) to measure the production of NADPH in 1 ml reaction mixtures containing 2 mM glucose 6-phosphate, 0.3 mM NADP⁺ and the cell extract.

RESULTS

Induction of the *zwf* genes in the presence of various carbon sources

There are three putative homologues of *zwf* (*zwf-1*, *zwf-2* and *zwf-3*) in the *P. putida* KT2440 genome (Fig. 2a, b, c).

Fig. 2. Genes involved in glucose catabolism in *P. putida* KT2440. The *zwf-1* (a) gene homologues are *zwf-2* (b) and *zwf-3* (c). The complete genome sequence of *P. putida* KT2440 in the NCBI database was the source for the gene numbers and organization.

In the presence of various sources of carbon, we investigated their expression using Northern blot analysis. Total RNAs were isolated from 3 ml exponentially growing cells. The level of *zwf-1* transcription, as determined by Northern blotting, increased dramatically when *P. putida* KT2440 was cultured in minimal medium containing either glucose or gluconate as the sole carbon source. However, in minimal medium supplemented with either pyruvate or succinate, the level of *zwf-1* gene induction was very low and likely represented a basal level (data not shown). The expression levels of *zwf-2* and *zwf-3* were constitutive, and they remained low regardless of the carbon source (data not shown). These results indicate that *zwf-2* and *zwf-3* play minor roles in glucose metabolism, whereas the *zwf-1* gene product is the main enzyme required for converting glucose 6-phosphate to 6-phosphogluconate. Induction of the *zwf-1* gene occurred when glucose or gluconate metabolism was required,

whereas the organic acids pyruvate and succinate had no apparent effect on *zwf-1* induction.

HexR represses *zwf-1* induction

The mechanism of HexR regulation in *P. putida* KT2440 was investigated using a reporter plasmid that contained the *gfp* gene under the control of P_{zwf-1} . The reporter plasmid was introduced into either the wild-type or the HexR knockout mutant strain (HexR⁻) to generate *P. putida* KT2440 (pRK_{*zwf-1gfp*}) and *P. putida* KT2440 (HexR⁻/pRK_{*zwf-1gfp*}). Consistent with the results of the Northern blot assay, the reporter assay indicated that P_{zwf-1} activity increased significantly in minimal medium supplemented with either glucose or gluconate compared with its activity in minimal medium containing either pyruvate or succinate (Fig. 3). The GFP expression levels in minimal medium containing glucose or gluconate were 25–50-fold

Fig. 3. Quantification of GFP expression in wild-type and HexR- reporter strains grown in the presence of various carbon sources. GFP was measured as described in Methods. Grey bars, *P. putida* KT2440 (pRKp_{zwf-1gfp}); black bars, HexR- (pRKp_{zwf-1gfp}); white bars, HexR- (pRKp_{zwf-1gfp}-HexR CP).

higher than those observed in pyruvate- or succinate-containing minimal medium. The P_{zwf-1} activity in either glycerol- or fructose-containing minimal medium was much higher than in either the pyruvate- or succinate-containing medium, but it remained at only half of the activity level observed in the glucose-containing medium (Fig. 3). When these results are taken together, the conclusion appears to be that the *zwf-1* gene is inducible, and that this occurs when metabolism of glucose, gluconate, glycerol or fructose is required. However, in the HexR- strain the GFP activity was constitutively high under all conditions (Fig. 3, black bars). The levels of P_{zwf-1} activity were similar in wild-type and HexR-complementation strains (Fig. 3, white bars) under all conditions tested. Collectively, it appears that HexR regulates *zwf-1* induction as a repressor.

To investigate whether HexR binds to the *zwf-1* promoter region, EMSAs were conducted using a P_{zwf-1} DNA probe (−136 to +34 of the *zwf-1* promoter region) with cell extracts from *E. coli* BL21 harbouring a HexR-overexpression vector. The EMSA results demonstrated that a crude extract of *E. coli* BL21 [pET28a(+)-HexR] cells retards the migration of the P_{zwf-1} DNA probe (Fig. 4a). The EMSA data indicated that a HexR operator site might lie in the *zwf-1* promoter-specific region.

The *zwf-1* promoter region contains a HexR operator site

To examine the HexR binding regions, the 221 P_{zwf-1} , 136 P_{zwf-1} , 67 P_{zwf-1} and 19 P_{zwf-1} variants of the *zwf-1* promoter were constructed (kt *zwf-1* sp. pro3, kt P_{zwf-1} pt1, pt2, pt3 as upper primer, respectively, and kt *zwf-1* pro2 as a lower primer; Table 2, Fig. 4b). Potential binding regions of differing sizes were cloned into the *gfp* reporter vector (pRK415gfp) and were introduced into the wild-type and

HexR- strains. The 221 P_{zwf-1} yielded the highest levels of GFP expression in gluconate-containing minimal medium, whereas GFP did not accumulate in the succinate-containing minimal medium (Fig. 4c). The 136 P_{zwf-1} behaved similarly to 221 P_{zwf-1} (Fig. 4c). However, the 136 P_{zwf-1} activity was twofold lower than that of 221 P_{zwf-1} in gluconate or succinate, regardless of the presence of HexR (Fig. 4c). Due to the absence of the RNA polymerase binding site, there was no 19 P_{zwf-1} activity (Fig. 4c). Interestingly, we found that the reporter strain containing 67 P_{zwf-1} expressed GFP in the succinate medium (Fig. 4c), and similar levels of GFP were observed in the HexR- reporter strain carrying 67 P_{zwf-1} in minimal medium supplemented with either succinate or gluconate (Fig. 4c). Together, these data provide evidence that: (1) the region between −67 and −136 is important for HexR binding, (2) 67 P_{zwf-1} is not involved in the regulation of HexR, and (3) the region far upstream of the HexR binding site might be essential for full expression of the *zwf-1* gene.

KDPG is a real inducer of Hex operons

It has been suggested that 6PG is the inducer of the Hex operon (Petruschka *et al.*, 2002; Wu & Weiss, 1992). However, our data show that 6PG does not inhibit the binding of HexR to the *zwf-1* promoter region. Instead, KDPG influences the binding of HexR to its cognate promoter region (Fig. 5a). The EMSA assay showed that binding of HexR to the *zwf-1* promoter region is blocked by KDPG but not by 6PG.

The *edd* and *eda* deletion strains were used to confirm that KDPG is a real inducer of HexR *in vivo*. The *edd* mutant cannot make KDPG, and the *eda* mutant accumulates KDPG inside cells. Wild-type cells and mutants bearing the pRKp_{zwf-1gfp} reporter plasmid were precultured in 10-fold diluted LB medium until they reached exponential growth

Fig. 4. (a) EMSA of the *zwf-1* promoter region probe with cell extracts from BL21 (HexR). Lane 1, free *zwf-1* promoter region probe; lanes 2–6, 0.25, 0.5, 1, 5 and 10 µg BL21 (HexR) cell extract, respectively; lane 7, BL21 cell extracts harbouring pET28. (b) Schematic representation of the 5'-deleted *zwf-1* promoter region fused to the *gfp* reporter plasmid. The transcription start point is taken from del Castillo *et al.* (2008). (c) Quantification of GFP expression in wild-type and HexR-derivative reporter strains grown with gluconate or succinate.

phase and were transferred to minimal medium containing either gluconate or succinate. After a 1 h incubation, we measured the level of GFP induction. The P_{zwf-1} activity of the *eda* mutant increased dramatically even in the succinate-amended conditions (Fig. 5b). However, the GFP activity of the *edd* mutant remained low in the gluconate-amended medium (Fig. 5b). As such, it appears that KDPG is the inducer of *zwf-1* expression.

The *zwf-1* gene is also induced by an oxidative stress condition

Bacteria may protect themselves against oxidative stress by using enzymes that include superoxide dismutase, catalase, glutathione/thioredoxin peroxidase and/or G6PDH to scavenge reactive oxygen species (ROS) (Halliwell &

Gutteridge, 1999). We have previously shown that the *zwf-1* gene in *P. putida* KT2440 is induced by oxidative stress in response to reagents such as PQ or MD (Park *et al.*, 2006). To investigate the response of each of the *zwf* gene homologues to oxidative stress, Northern blot analysis was performed in the presence of PQ, MD, As or cumene hydroperoxide (CHP). Each of these reagents is able to produce superoxide or hydroxyl radicals under aerobic conditions (Gant *et al.*, 1998; Parvatiyar *et al.*, 2005). Under these conditions, the *zwf-2* and *zwf-3* induction levels were negligible (Fig. 6a); however, the *zwf-1* gene was highly expressed under all conditions except in the presence of PQ (Fig. 6a, b). The *zwf-1* gene was the only oxidative stress-related gene that responded to these reagents. Similar levels of induction were observed when the G6PDH activities were measured after a 30 min

Fig. 5. (a) EMSA of the *P_{zwf-1}* probe using BL21 (HexR) crude extracts (5 μ g), KDPG or 6PG. The first lane contains the free *zwf-1* promoter region probe. (b) *P_{zwf-1}* activity in wild-type, Edd and Eda mutant strains. Each strain was precultured in 10-fold-diluted LB medium until growth reached the exponential phase (OD_{600} 0.3–0.4), and cells were harvested, washed with PBS, and divided into gluconate or succinate medium. GFP intensity was determined after 1 h.

exposure to PQ (1 mM), MD (1 mM) or As (10 p.p.m.). With As and MD, the activity ($U\ mg^{-1}$) was 0.6 ± 0.05 and 0.517 ± 0.02 , respectively. The level of G6PDH activity was similar with PQ (0.375 ± 0.04) and under control (0.375 ± 0.01) conditions, probably because of the low level of *zwf-1* expression.

HexR may directly detect oxidative stress

To demonstrate the existence of an additional activation system, EMSA was conducted using crude extracts of wild-type and HexR $^-$ cells. Proteins in the HexR $^-$ cell crude extracts (25 μ g) did not bind to the *zwf-1* promoter region, and gel-shift retardation occurred in the crude extracts from wild-type (25 μ g) and BL21 (HexR) (and HexR $^-$ cells) (Fig. 7a). When the full-length promoter regions were used to rule out the possibility of an upstream activator protein, we observed the same result (Fig. 7a). If there was an additional system for *zwf-1* gene induction, then during exposure to oxidative stress the level of *zwf-1* transcription in the wild-type strains would exceed that of the HexR $^-$ strain. However, Northern blot analyses indicated that the levels of *zwf-1* induction in the HexR $^-$ strain were almost identical in the presence or absence of oxidative stress reagents (Fig. 7b).

Thus, it is highly probable that the only operative oxidative stress system involves HexR-regulated *zwf-1* activation. These results suggest that HexR is the only protein able to bind to the *zwf-1* promoter region, and that HexR is a good candidate for the oxidative stress-sensing regulator of the *zwf-1* promoter.

We also investigated the indirect induction of the *zwf-1* gene by HexR, under the conditions of oxidative stress. If KDPG were overproduced, then high levels of *zwf-1* expression could occur, through an unknown mechanism, under conditions of oxidative stress. To confirm this possibility, Northern blot analysis was performed using the Δ Edd and Δ Eda strains. The Δ Edd strain expressed the *zwf-1* gene in response to As exposure in the absence of KDPG (Fig. 7c). Consistent with the results of the GFP-based reporter assay, a high level of *zwf-1* expression was observed in the Δ Eda strain cultured in LB medium with or without As exposure (Fig. 7c). Thus, it seems unlikely that KDPG is required for *zwf-1* gene induction under oxidative stress conditions. This observation also confirmed that there was no other activation system for *zwf-1* induction in the presence of oxidative stress reagents (Fig. 7c). This observation is further supported by the fact

Fig. 6. Induction of the *zwf* homologue genes (*zwf-1*, *zwf-2* and *zwf-3*) exposed to oxidative stress. Total mRNA was extracted 1, 5 or 10 min after treatment of *P. putida* KT2440 with (a) 1 mM PQ, 0.5 mM MD, 10 p.p.m. As or (b) 5 mM CHP. C, control.

that wild-type cells exhibited higher *zwf-1* expression in response to oxidative stress in the succinate-amended minimal medium (Fig. 7d).

Recently, del Castillo and colleagues have reported that HexR controls *edd* as well as *gap-1* in *P. putida* (Fig. 1; del Castillo *et al.*, 2008). This implies that the *edd* and *gap-1* promoter regions contain HexR operator sites, and that the *edd* and *gap-1* genes are regulated by the HexR repressor. If HexR is a direct sensor of oxidative stress, then there should be an increase in *edd* expression with oxidative stress. The results revealed a high level of *edd* gene expression under oxidative stress conditions (Fig. 7e). As in HexR- and KDPG-related *zwf-1* gene induction, the *edd* gene was also induced in the HexR- and ΔEda strains due to the absence of the HexR repressor and the presence of the HexR inducer, respectively (Fig. 7f). It seems that the induction of oxidative stress via the *edd* promoter region occurs as a result of more complex mechanisms.

In *P. aeruginosa* PAO1, a locus designated *hexC* (129 bp, *cis*-element) exists between the *gap* and *edd* genes (Temple *et al.*, 1994). This region reportedly contains binding sites not only for HexR but also for the integration-host factor (IHF; Proctor *et al.*, 1997). We conducted an *in vitro* EMSA analysis to examine whether HexR is a direct sensor of oxidative stress. The data showed that binding of HexR to its cognate promoter was inhibited by CHP (50 mM) and MD (20 mM; data not shown). Although these concentrations may not be physiologically relevant, we chose them

because high concentrations of other non-specific proteins and reagents such as hydroperoxide (500 mM) and *tert*-butyl hydroperoxide (500 mM) did not inhibit HexR binding in crude extracts (data not shown). Although the precise mechanism remains unknown, we have provided clear evidence that HexR may directly detect oxidative stress.

HexR- deletion improves tolerance to oxidative stress

The tolerance to oxidative stress was compared in wild-type and HexR- cells. In the presence of MD or PQ, the HexR- strain was less sensitive than the wild-type cells (Fig. 8). The HexR- strain might be more resistant to oxidative stress due to the constitutive activity of G6PDH. However, exposure to As did not result in differential sensitivity of the wild-type and HexR- strains to stress (Fig. 8). It is possible that *zwf-1* expression in response to As exposure might be sufficiently rapid and strong to defend against oxidative stress (Fig. 6).

DISCUSSION

G6PDH is required in the ED pathway of *Pseudomonas* species. NADPH, produced by G6PDH, is essential for defence against oxidative stress. Interestingly, the *P. putida* KT2440 genome contains three homologues of *zwf*. The amino acid identities of *zwf-1* to *zwf-2* and of *zwf-1* to *zwf-3* are 36.8% and 48.2%, respectively. Northern blot analysis showed that *zwf-2* and *zwf-3* do not play major roles in the ED pathway, but that the *zwf-1* gene is a key enzyme in this pathway and is also important for defence against oxidative stress (Fig. 6a, b). Our results showed that, under conditions of oxidative stress, the *zwf-1* gene is induced, whereas the levels of *zwf-2* and *zwf-3* expression do not change (Fig. 6a). Although the NCBI database shows that various micro-organisms contain more than one *zwf* gene, the functions of the additional genes and of G6PDH remain unclear. Nevertheless, only *zwf-1* gene products responded to glucose metabolism in *P. putida*. *Deinococcus radiophilus* carries the G6PDH-1 and G6PDH-2 isoforms of G6PDH (Sung & Lee, 2007). When *D. radiophilus* was treated with potassium superoxide, the activity of G6PDH-1 increases markedly, but the G6PDH-2 activity does not increase (Sung & Lee, 2007). The functions of *zwf-2* and *zwf-3* in *P. putida* remain to be investigated.

The data presented demonstrated that HexR binds to the promoter regions of the *zwf-1* gene and functions as a repressor. Because the full-length promoter expresses *zwf-1* at levels approximately twofold higher than those observed with promoters of various lengths, there may be more complex mechanisms of *zwf-1* gene regulation (Fig. 4c). It may be that the upstream region (−221 to −136) contains *cis* elements that are important for the full induction of *zwf-1* transcription. Many research groups have proposed

Fig. 7. Investigation of the *zwf-1* gene activation system. (a) EMSA of the *P_{zwf-1}* probe (221 and 136 bases upstream of the *zwf-1* transcription start point) using cell extracts from wild-type (25 µg), HexR- (25 µg) and BL21 (HexR) (10 µg) strains. (b) Northern blot analysis of the *zwf-1* gene in HexR- bacteria exposed to 1 mM PQ, 0.5 mM MD and 10 p.p.m. As. Cells were exposed to each substrate for 1 min. (c) Northern blot analysis of the *zwf-1* gene in wild-type cells grown in minimal medium with succinate then exposed to As (1, 5 and 10 p.p.m.) for 1 and 10 min. (d) Northern blot analysis of the *zwf-1* gene in wild-type cells that were exposed to 0.5 mM MD, 10 p.p.m. As or 5 mM CHP. Cells were exposed to each substrate for 1 or 10 min (CHP). (e, f) Northern blot analysis of the *zwf-1* (c) or *edd* (f) genes in different mutant strains exposed to As (10 p.p.m.) for 1 min. C, control.

that 6PG is an inducer of HexR; however, our results demonstrated that HexR binding is inhibited by KDPG rather than by 6PG (Fig. 5a). In *P. aeruginosa*, KDPG may physiologically induce repressed genes, including *edd*, *gap* and *zwf* (Temple *et al.*, 1998). However, to our knowledge, there are no data to support this idea. Interestingly, it has recently been reported that KDPG, generated during glucose utilization, is also a chemical signal involved in toluene catabolic repression in *P. putida* KT2440 (del Castillo & Ramos, 2007a). Previous data, along with our data, have demonstrated that KDPG might be a very important regulatory intermediate for controlling efficient energy metabolism.

Because it was very difficult to purify the HexR proteins, we used crude extracts harbouring overexpressed HexR throughout our *in vitro* experiments. The difficulty of purifying HexR is caused by its complex polymer structure. Although we used crude extracts containing His-tagged HexR, the same results were obtained with crude extracts containing wild-type HexR (data not shown). We strongly believe that there is no difference between His-tagged HexR and wild-type HexR in terms of both their binding to the cognate DNA region and their sensing KDPG and oxidative stress. Although the structure of HexR has not been determined, the NCBI database shows that HexR proteins feature a helix–turn–helix domain in their N-terminal

Fig. 8. Sensitivity of wild-type and HexR[−] strains to oxidative stress. The strains were cultured until they reached the exponential growth phase, serially diluted and spotted onto LB agar supplemented with various inducers of oxidative stress.

region and a sugar isomerase (SIS) domain in their C-terminal region. The SIS domain functions in phospho-sugar binding, and KDPG might bind to the HexR-SIS domain, alter its conformation, and inhibit its binding to DNA.

ROS, such as the superoxide anion radical and its derivatives hydrogen peroxide and the hydroxyl radical, can damage bacterial cells (Girò *et al.*, 2006). To protect itself against superoxide and nitric oxide stresses, *E. coli* has the SoxR regulon (Dempsey, 1996; Park *et al.*, 2006). Under normal conditions, the [2Fe–2S] centre in SoxR exists in a reduced state. If this iron–sulfur cluster is oxidized by ROS, the SoxR is activated, and SoxS expression is induced. In *E. coli*, SoxS is known to be a transcriptional activator for *zwf* expression. The defence role of G6PDH against oxidative stress has been described before (Ceccarelli *et al.*, 2004; Lundberg *et al.*, 1999). Although a SoxR homologue is present, SoxS is absent from *P. putida*, and little is known about the scavenging mechanism in *Pseudomonas* species that is induced by oxidative stress (Park *et al.*, 2006). The *fpr* gene, which encodes ferredoxin-NADP⁺ reductase in *P. putida*, is involved in oxidative stress defence and its expression is regulated by FinR (Lee *et al.*, 2006b). However, the expression of *fpr* in *E. coli* occurs under the control of SoxS. The system regulating *zwf-1* induction under conditions of oxidative stress in *P. putida* remains unknown.

To our knowledge, this is the first report to demonstrate that HexR detects ROS and controls *zwf-1* expression under the conditions of oxidative stress. Our conclusions are strongly supported by the findings that oxidative stress (1) induces the *zwf-1* gene, despite the absence of intracellular KDPG; (2) inhibits HexR binding to its cognate promoter regions; and (3) strongly induces the *edd* gene, which does not appear to play a significant role in oxidative stress defence. Because some oxidative stress-inducing reagents strongly induced *zwf-1* expression, but

did not inhibit HexR binding to its cognate promoter, there may be a more complex mechanism through which HexR detects oxidative stress (data not shown).

ACKNOWLEDGEMENTS

This work was supported by a NCRC (National Core Research Center) grant (R15-2003-002-01002-0) and a Korea Science and Engineering Foundation (KOSEF) grant (R01-2008-000-10697-0) to W.P. This work was also supported by a Korea University Grant (2008). We thank Matthew J. Walters (Dr Eric J. Toone's laboratory, Department of Chemistry, Duke University) for providing the KDPG substrate.

REFERENCES

- Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A. & Struhl, K. (1999). *Current Protocols in Molecular Biology*. New York: Wiley & Sons.
- Ceccarelli, E. A., Arakaki, A. K., Cortez, N. & Carrillo, N. (2004). Functional plasticity and catalytic efficiency in plant and bacterial ferredoxin–NADP(H) reductases. *Biochim Biophys Acta* **1698**, 155–165.
- Cuskey, S. M., Wolff, J. A., Phibbs, P. V. & Olsen, R. H. (1985). Cloning of genes specifying carbohydrate catabolism in *Pseudomonas aeruginosa* and *Pseudomonas putida*. *J Bacteriol* **162**, 865–871.
- del Castillo, T. & Ramos, J. L. (2007a). Simultaneous catabolic repression between glucose and toluene metabolism in *Pseudomonas putida* is channeled through different signaling pathways. *J Bacteriol* **189**, 6602–6610.
- del Castillo, T., Ramos, J. L., Rodríguez-Herva, J. J., Fuhrer, T., Sauer, U. & Duque, E. (2007b). Convergent peripheral pathways catalyze initial glucose catabolism in *Pseudomonas putida*: genomic and flux analysis. *J Bacteriol* **189**, 5142–5152.
- del Castillo, T., Duque, E. & Ramos, J. L. (2008). A set of activators and repressors control peripheral glucose pathway in *Pseudomonas putida* to yield a common central intermediate. *J Bacteriol* **190**, 2331–2339.
- Dempsey, B. (1996). Redox signaling and gene control in the *Escherichia coli* soxRS oxidative stress regulon – a review. *Gene* **179**, 53–57.

- Duque, E., Molina-Henares, A. J., de la Torre, J., Molina-Henares, M. A., del Castillo, T., Lam, J. & Ramos, J. L. (2007). Towards a genome-wide mutant library of *Pseudomonas putida* strains KT2440. In *Pseudomonas*, vol. V, chapter 8, pp. 227–251. Edited by J. L. Ramos & A. Filloux. Dordrecht, The Netherlands: Springer.
- Fredrickson, J. K., Bezdicek, D. F., Brockman, F. J. & Li, S. W. (1988). Enumeration of Tn5 mutant bacteria in soil by using a most-probable-number–DNA hybridization procedure and antibiotic resistance. *Appl Environ Microbiol* **54**, 446–453.
- Gant, T. W., Rao, D. N., Mason, R. P. & Cohen, G. M. (1988). Redox cycling and sulphhydryl arylation; their relative importance in the mechanism of quinine cytotoxicity to isolated hepatocytes. *Chem Biol Interact* **65**, 157–173.
- Girò, M., Carrillo, N. & Krapp, A. R. (2006). Glucose-6-phosphate dehydrogenase and ferredoxin-NADP(H) reductase contribute to damage repair during *soxRS* response of *Escherichia coli*. *Microbiology* **152**, 1119–1128.
- Greenberg, J. T., Monach, P., Chou, J. H., Josephphy, P. D. & Demple, B. (1990). Positive control of a global antioxidant defense regulon activated by superoxide-generating agents in *Escherichia coli*. *Proc Natl Acad Sci U S A* **87**, 6181–6185.
- Hager, P. W., Calfee, M. W. & Phibbs, P. V. (2000). The *Pseudomonas aeruginosa* *devB*/SOL homolog, *pgl*, is a member of the *hex* regulon and encodes 6-phosphogluconolactonase. *J Bacteriol* **182**, 3934–3941.
- Halliwell, B. & Gutteridge, J. M. C. (1999). Antioxidant defenses. In *Free Radicals in Biology and Medicine*, 3rd edn, pp. 105–159. Oxford, UK: Oxford Science Publications.
- Hidalgo, E., Ding, B. & Demple, B. (1997). Redox signal transduction: mutations shifting [2Fe–2S] centers of the SoxR sensor-regulator to the oxidized form. *Cell* **88**, 121–129.
- Kalogeraki, V. S. & Winans, S. C. (1997). Suicide plasmids containing promoterless reporter genes can simultaneously disrupt and create fusions to genes of diverse bacteria. *Gene* **188**, 69–75.
- Lee, Y., Ahn, E., Park, S., Madsen, E. L., Jeon, C. O. & Park, W. (2006a). Construction of a reporter strain *Pseudomonas putida* for the detection of oxidative stress caused by environmental pollutants. *J Microbiol Biotechnol* **16**, 386–390.
- Lee, Y., Peña-Llopis, S., Kang, Y. S., Shin, H.-D., Demple, B., Madsen, E. L., Jeon, C. O. & Park, W. (2006b). Expression analysis of the *fpr* (ferredoxin-NADP⁺ reductase) gene in *Pseudomonas putida* KT2440. *Biochem Biophys Res Commun* **339**, 1246–1254.
- Li, Z. & Demple, B. (1994). SoxS, an activator of superoxide stress gene in *Escherichia coli*. *J Biol Chem* **269**, 18371–18377.
- Lundberg, B., Wolf, R. E., Dinuer, M., Xu, Y. & Fang, F. (1999). Glucose 6-phosphate dehydrogenase is required for *Salmonella typhimurium* virulence and resistance to reactive oxygen and nitrogen intermediates. *Infect Immun* **67**, 5371–5375.
- Ma, J. F., Hager, P. W., Howell, M. L., Phibbs, P. V. & Hassett, D. J. (1998). Cloning and characterization of the *Pseudomonas aeruginosa* *zwf* gene encoding glucose-6-phosphate dehydrogenase, an enzyme important in resistance to methyl viologen (paraquat). *J Bacteriol* **180**, 1741–1749.
- Park, W., Jeon, C. O., Hohnstock-Ashe, A. M., Wnas, S. C., Zylstra, G. J. & Madsen, E. L. (2003). Identification and characterization of the conjugal transfer region of the pCg1 plasmid from naphthalene-degrading *Pseudomonas putida* Cg1. *Appl Environ Microbiol* **69**, 3263–3271.
- Park, W., Peña-Llopis, S., Lee, Y. & Demple, B. (2006). Regulation of superoxide stress in *Pseudomonas putida* KT2440 is different from SoxR paradigm in *Escherichia coli*. *Biochem Biophys Res Commun* **341**, 51–56.
- Parvatiyar, K., Alsabbagh, E. M., Ochsner, U. A., Stegemeyer, M. A., Smulian, A. G., Hwang, S. H., Jackson, C. R., Mcdermott, T. R. & Hassett, D. J. (2005). Global analysis of cellular factors and responses involved in *Pseudomonas aeruginosa* resistance to arsenite. *J Bacteriol* **187**, 4853–4864.
- Petruschka, L., Adolf, K., Burchhardt, G., Dervede, J., Jürgensen, J. & Herrmann, H. (2002). Analysis of the *zwf-pgl-eda*-operon in *Pseudomonas putida* strains H and KT2440. *FEMS Microbiol Lett* **215**, 89–95.
- Pomposiello, P. J. & Demple, B. (2001). Redox-operated genetic switches: the SoxR and OxyR transcription factors. *Trends Biotechnol* **19**, 109–114.
- Proctor, W. D., Arora, S., Hager, P. & Phibbs, P. V., Jr (1997). Integration host factor and the putative repressor protein *hexR* bind the *hexC* locus of *Pseudomonas aeruginosa*. *Abstracts of the 97th General Meeting of the American Society for Microbiology K-95*, p. 357. Washington DC: American Society for Microbiology
- Simon, R., Priefer, U. & Pufler, A. (1983). A broad host range mobilization system in Gram-negative bacteria. *Biotechnology (N Y)* **1**, 784–791.
- Singh, R., Beriault, R., Middaugh, J., Hamel, R., Chenier, D., Appanna, V. D. & Kalyuzhnyi, S. (2005). Aluminum-tolerant *Pseudomonas fluorescens*: ROS toxicity and enhanced NADPH production. *Extremophiles* **9**, 367–373.
- Sung, J. Y. & Lee, Y. N. (2007). Isoforms of glucose 6-phosphate dehydrogenase in *Deinococcus radiophilus*. *J Microbiol* **45**, 318–325.
- Temple, L., Cuskey, S. M., Perkins, R. E., Bass, R. C., Morales, N. M., Christie, G. E., Olsen, R. H. & Phibbs, P. V. (1990). Analysis of cloned structural and regulatory genes for carbohydrate utilization in *Pseudomonas aeruginosa* PAO. *J Bacteriol* **172**, 6396–6402.
- Temple, L., Sage, A., Christie, G. E. & Phibbs, P. V. (1994). Two genes for carbohydrate catabolism are divergently transcribed from a region of DNA containing the *hexC* locus in *Pseudomonas aeruginosa* PAO1. *J Bacteriol* **176**, 4700–4709.
- Temple, L., Sage, A., Schweizer, H. P. & Phibbs, P. V. (1998). Carbohydrate catabolism in *Pseudomonas aeruginosa*. In *Biotechnology Handbooks*, vol. 10, chapter 2, pp. 35–72. Edited by T. C. Montie. New York: Plenum.
- Tsaneva, I. R. & Weiss, B. (1990). *soxR*, a locus governing a superoxide response regulon in *Escherichia coli* K-12. *J Bacteriol* **172**, 4197–4205.
- Wu, J. & Weiss, B. (1992). Two-stage induction of the *soxRS* (superoxide response) regulon of *Escherichia coli*. *J Bacteriol* **174**, 3915–3920.
- Yin, S., Fuanthong, M., Laratta, W. P. & Shapleigh, J. P. (2003). Use of a green fluorescent protein-based reporter fusion for detection of nitric oxide produced by denitrifiers. *Appl Environ Microbiol* **69**, 3938–3944.

Edited by: M. A. Kertesz